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13. SUPPLEMENTARY NOTES

14. ABSTRACT:
We have developed a 'knock-in' mouse model with a mutation (glycine 525 to leucine, G525L) in estrogen receptor alpha (ERα) that permits exogenous regulation of its ligand-induced signaling pathways, while not affecting ligand-independent signaling. This ligand-binding pocket mutation significantly reduces ERα response to endogenous estrogens but not to the synthetic nonsteroidal estrogen diethylstilbestrol (DES). Therefore, ERα signaling pathways can be regulated in these mice through DES administration or withdrawal. Female mutant G525L ERα homozygous mice had immature and hypoplastic uterine and vaginal tissues and only developed rudimentary mammary gland ductal trees. Homozygous ovarian tissues had a hyperplasic stroma and no corpora lutea. In addition, some of the homozygous ovaries contained large, hemorrhagic, cystic follices. Cyst development increased with age. Homozygous animals had higher estrogen (E) and luteinizing hormone (LH) serum levels than their wild type and heterozygous littermates. Homozygous animals were also significantly larger than their wild type and heterozygous littermates. This was partly due to an increase in gonadel and mammary fat pad weight. Further analysis of this knock-in mouse model, including examination of non-reproductive tissues and uterotrophic assays with E, DES, and epidermal growth factor (EGF), will provide valuable information about the role of ligand-induced and ligand-independent ERα signaling in development and carcinogenesis.

15. SUBJECT TERMS
Estrogen Receptor, Steroid Receptor, Endocrine Signaling, Estrogen

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INTRODUCTION

Estrogen receptor alpha (ERα) is a crucial therapeutic target for hormone dependent breast cancers. More effective treatment and prevention strategies are likely to emerge from an improved understanding of ERα mechanisms *in vivo*. To achieve this goal, we have developed a 'knock-in' mouse model with a mutation in ERα (glycine 525 to leucine, G525L) that permits exogenous regulation of its ligand-induced signaling pathways, while not affecting ligand-independent signaling. This ligand-binding pocket mutation significantly reduces ERα response to endogenous estrogens but not to the synthetic nonsteroidal estrogen diethylstilbestrol (DES). Therefore, ERα signaling pathways can be regulated in these mice through DES administration or withdrawal. These activities can be regulated both in developing animals as well as in adult animals exposed to tumorigenic agents, providing valuable information about the role of ERα in mammary gland development and carcinogenesis.

BODY

Task 1: To define the contribution of classical ERα activation in murine mammary gland development.

1. Analyze the mutant G525L ERα knock-in mouse phenotype

a. Sequencing of genomic DNA confirmed the G525L mutation was present in homozygous animals. A reverse transcriptase polymerase chain reaction (RT-PCR) strategy is currently being developed to confirm transgene expression. RT-PCR can be used to quantify mRNA levels from smaller samples than an RNase protection assay and should provide better results. The primers have been developed and tested on wild type RNA. Uterine tissues from wild type, heterozygous, and homozygous animals have been frozen for future assays. Uterine ERα protein levels were analyzed by Western blots (Figure 1). There were equivalent levels of ERα in the wild type, heterozygous, and homozygous uterine tissues. In addition, mutant G525L ERα was detected in heterozygous and homozygous uterine tissues with the 6xHis-tag antibody (Figure 1).

b. To evaluate the phenotype of all potential sites of mutant G525L ERα expression, reproductive tissues from 6-, 12-, and 20-week-old female mice were analyzed. Luciferase assays with Ishikawa cells, cotransfected with mutant G525L ERα and an estrogen response element (ERE)-reporter, showed transcription was not stimulated by low concentrations of E2, but was stimulated by DES or genistein (Figure 2). Since genistein is a phytoestrogen and may stimulate the mutant G525L ERα *in vivo*, half the animals were placed on a soy-free diet. The phenotype between animals on a regular and soy-free diet will be compared. Preliminary results indicated 12- and 20-week-old homozygous animals on a regular and soy-free diet were significantly larger than their wild type and heterozygous littermates (Figure 3). This was partly due to an increase in gonadel and mammary fat pad weights (Figures 4 & 5). 12- and 20-week-old homozygous animals on a soy-free diet and 20-week-old animals on a regular diet had significantly larger gonadel and mammary fat pads than their wild type and heterozygous littermates. Thus, a soy-free diet accelerated this phenotype (Figures 4 & 5). Preliminary data indicated that animals on a regular and soy-free diet had similar reproductive tract

phenotypes. Homozygous uterine tissues had immature and hypoplastic uterine tissue and a lack of estrogenization of the luminal and glandular epithelium (Figure 6). Homozygous vaginal tissues lacked the estrogen-induced stratification and cornification seen in the wild type and heterozygous tissues (Figure 6). Homozygous ovaries had a hyperplasic stroma and no corpora lutea (Figure 7). In addition, some of the homozygous ovaries contained large, hemorrhagic, cystic follices (Figure 7). Severity of the homozygous phenotype increased with age. Female animals were infertile since they did not ovulate. Teresa Woodruff, a pathologist at Northwestern University, has agreed to analyze the ovary sections in more detail. Luteinizing hormone (LH), estradiol (E2), and testosterone (T) levels were elevated in the 12-week-old homozygous females (Figures 8, 9, & 10). Homozygous LH levels were 12 and 32 times higher than wild type levels, in animals on a regular and soy-free diet, respectively. Thus, the animals on a soy-free diet had a more severe phenotype. Homozygous E2 levels were 3.5 and 3.8 times higher than wild type levels in animals on a regular and soy-free diet, respectively (Figures 8 & 9). The T data is preliminary and additional serum samples need to be analyzed (Figure 10). Progesterone, follicle stimulating hormone, and prolactin levels will also eventually be measured. In the future, non-reproductive tissues like the bone, brain, and heart will be studied.

c. Since 3-week-old wild type, heterozygous, and homozygous animals all had a rudimentary ductal mammary gland tree, 6-, 12-, and 20-week-old animals were examined. Animals on a regular and soy-free diet had similar phenotypes. Mammary gland whole mounts of 6-week-old animals showed homozygous females had a rudimentary underdeveloped epithelial ductal tree, while wild type and heterozygous females had a ductal tree extending to the lymph node and enlarged terminal end buds (Figure 11). In 12-week-old animals, wild type and heterozygous ducts filled the entire mammary fat pad and had extensive branching and alveolar budding, while homozygous mammary glands did not develop beyond a rudimentary epithelial ductal tree (Figure 11). This indicated the homozygous mammary glands were unresponsive to estrogen. Barbara Susnick, a breast pathologist at Northwestern University, has agreed to analyze the differences between the wild type and heterozygous mammary gland whole mounts. In the future, mRNA and protein expression levels for ERα and progesterone receptor (PR) will be quantified

2. Determine the optimal DES dose for normal mammary gland development and fertility.

a. Immature mouse uterotrophic assays with E2 and DES were performed to confirm E2 would not stimulate the mutant G525L ERα receptor *in vivo* and DES would activate the receptor. For the uterotrophic assays, females of 18-21 days of age were subcutaneously injected with various doses of E2 or DES for three consecutive days and sacrificed on the morning of the fourth day[1]. The uterine wet weight to body weight ratio was determined as a measure of uterine estrogenic response[1]. Uterotrophic assays with purchased wild type animals were performed with 1, 10, 100, or 1000 ug/kg doses of E2 and DES to determine appropriate doses to use in experimental animals (Figure 12). Since there was a high uterotrophic response with both E2 and DES at 100 and 1000 ug/kg in wild type animals, experimental animals were treated with doses of 100, 1000, or 10000 ug/kg. The preliminary results indicated the homozygous animals did not

respond to any of the E2 doses (Figure 13). Surprisingly, there was only a slight uterotrophic response with a DES dose of 1000 or 10000 ug/kg in the homozygous animals (Figure 13). The reason why high doses of DES did not stimulate a uterotrophic response in the homozygous animals will be investigated. ERα and mutant G525L ERα protein levels will be measured to confirm there is expression of the mutant receptor. Additional tissues, including the mammary gland and ovary, will be analyzed to determine if this is a tissue specific effect. If there is no activation of the mutant G525L ERα receptor with DES in other tissues, one hypothesis is that low levels of DES may be needed to "prime" the receptor for activation (e.g. via upregulation of co-factors). Therefore, homozygous animals will be injected directly after birth with levels of DES that simulate levels of E2 found at this time point in normal animals and the uterotrophic assays will be repeated with these animals.

The remaining parts of task one and two will be completed in the future.

KEY RESEARCH ACCOMPLISHMENTS

- Homozygous mutant G525L ERα mutant mice were generated.
- Body, gonadel fat pad, and mammary gland weights were measured.
- The female reproductive tract and mammary gland phenotype of wild type, heterozygous, and homozygous 6-, 12-, and 20-week-old mice on a regular and soy-free diet were established.
- LH, E2, and T serum levels were measured in 12-week-old animals.

REPORTABLE OUTCOMES

Animal Model Generation:
ERα G525L knock-in mice

Oral Presentation:
Kerstin W. Sinkevicius, Karla A. Temple, Sonia L. Sugg, Fredric E. Wondisford, Kenneth S. Korach and Geoffrey L. Greene. Estrogen receptor alpha G525L knock-in mice. Cancer Biology Retreat, Delevan, WI, May 2005.

Poster Presentation:
Kerstin W. Sinkevicius, Karla A. Temple, Sonia L. Sugg, Fredric E. Wondisford, Kenneth S. Korach and Geoffrey L. Greene. Estrogen receptor alpha G525L knock-in mice. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA, June 2005.

Oral and Poster Presentation:
Kerstin W. Sinkevicius, Karla A. Temple, Sonia L. Sugg, Fredric E. Wondisford, Kenneth S. Korach and Geoffrey L. Greene. Estrogen receptor alpha G525L knock-in mice. Keystone Symposium on Nuclear Receptors: Steroid Sisters, Banff, Alberta, March 2006.

CONCLUSIONS

Phenotypic analysis of the mutant G525L ERα knock-in mice revealed ligand-induced ERα signaling is crucial in female murine reproductive tract development. Preliminary experiments indicated most of the reproductive tract phenotypes of these animals are similar to those of the ERα knock-out (αERKO) mice. Ovarian cyst development is delayed in the mutant G525L ERα knock-in mice, compared to the ERKO mice, indicating ligand-independent signaling may have a compensatory role. Uterotrophic assays with epidermal growth factor (EGF) and insulin-like growth factor (IGF) will be important in determining the role of cross-talk between ERα and growth hormone signaling pathways *in vivo*. Since ligand-independent signaling is hypothesized to be essential in non-reproductive tissues, analysis of the bone phenotype should be particularly interesting. Further analysis of this knock-in model will provide valuable information about the role of ligand-induced and ligand-independent ERα signaling in development and carcinogenesis. This information should facilitate the development of novel therapies for the treatment or prevention of breast cancer.

REFERENCES

1. Jefferson WN, Padilla-Banks E, Clark G, Newbold RR. Assessing estrogenic activity of phytochemicals using transcriptional activation and immature mouse uterotrophic responses. Journal of Chromatography. 2002;777:179-189.

APPENDICES

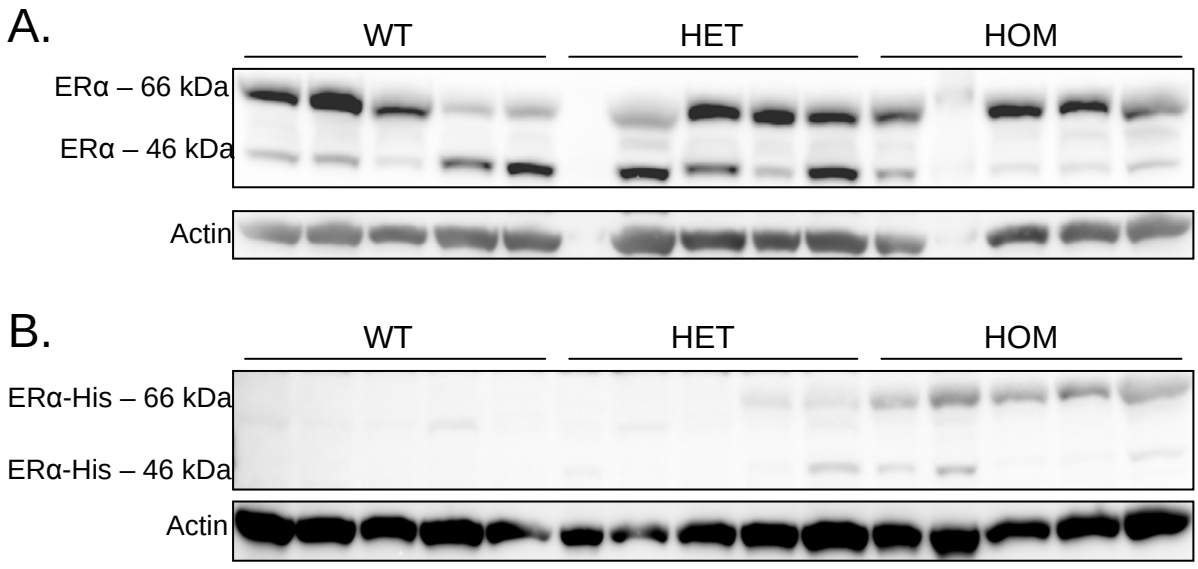

Figure 1. Western blot analysis of uterine protein extracts.
A: Western blot analysis of uterine protein extracts using an antibody to ERα.
B: Western blot analysis of uterine protein extracts using an antibody to 6xHis-tag.

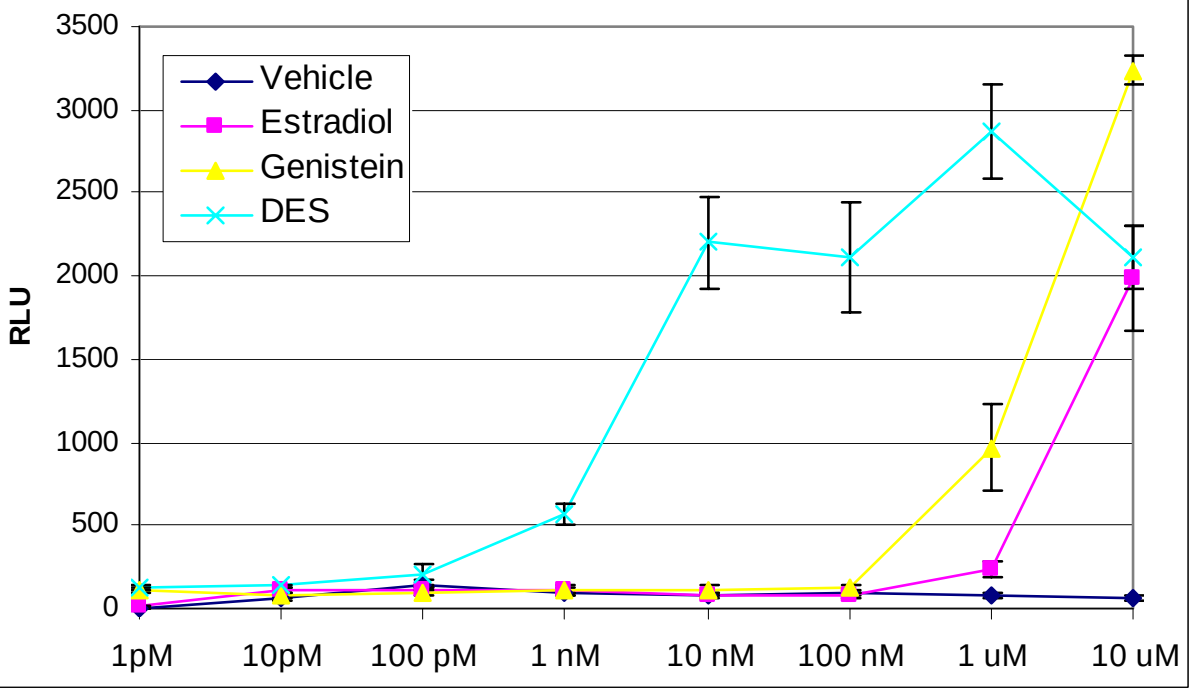

Figure 2. Transcriptional activation of mutant G525L ERα.
Luciferase assays with Ishikawa cells, cotransfected with mutant G525L ERα and an ERE-reporter, showed transcription was not stimulated by low concentrations of E2, but was stimulated by DES or genistein.

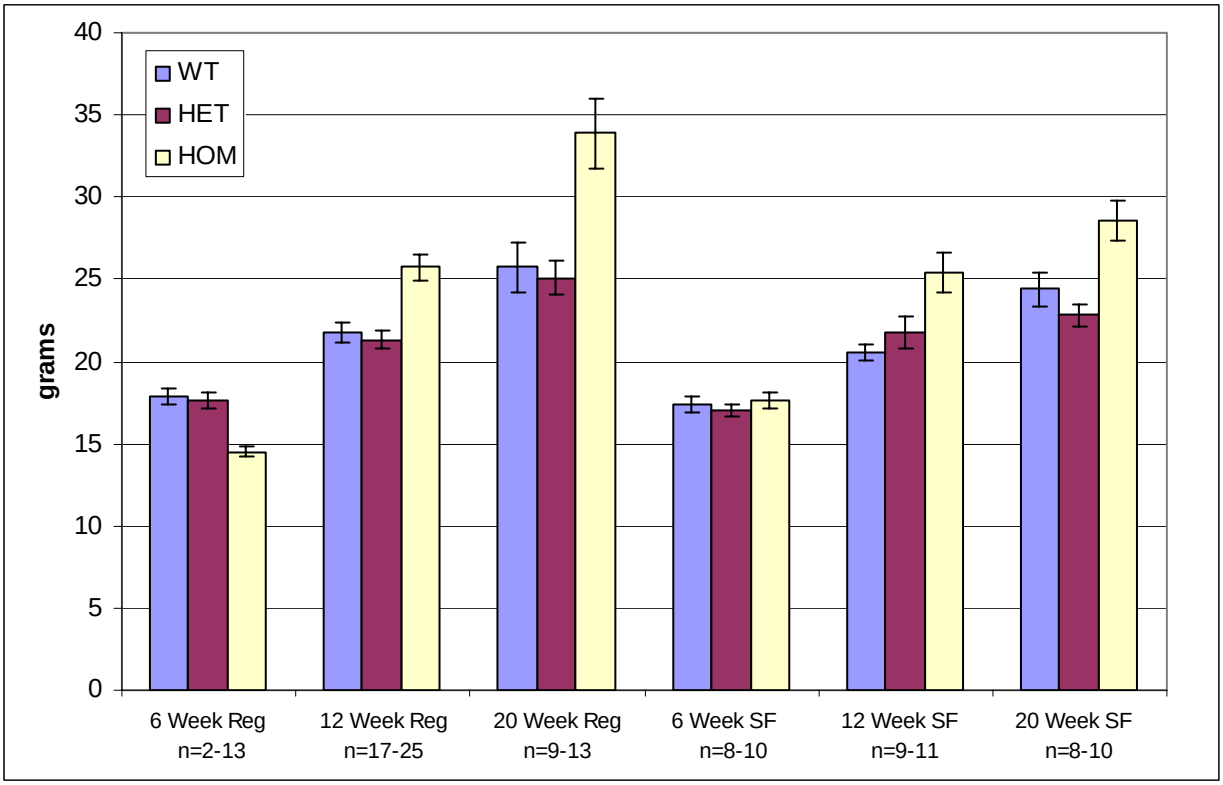

Figure 3. Female body weights.
6-, 12-, and 20-week-old wild type (WT), heterozygous (HET), and homozygous (HOM) animals on a regular (Reg) and soy-free (SF) diet were analyzed.

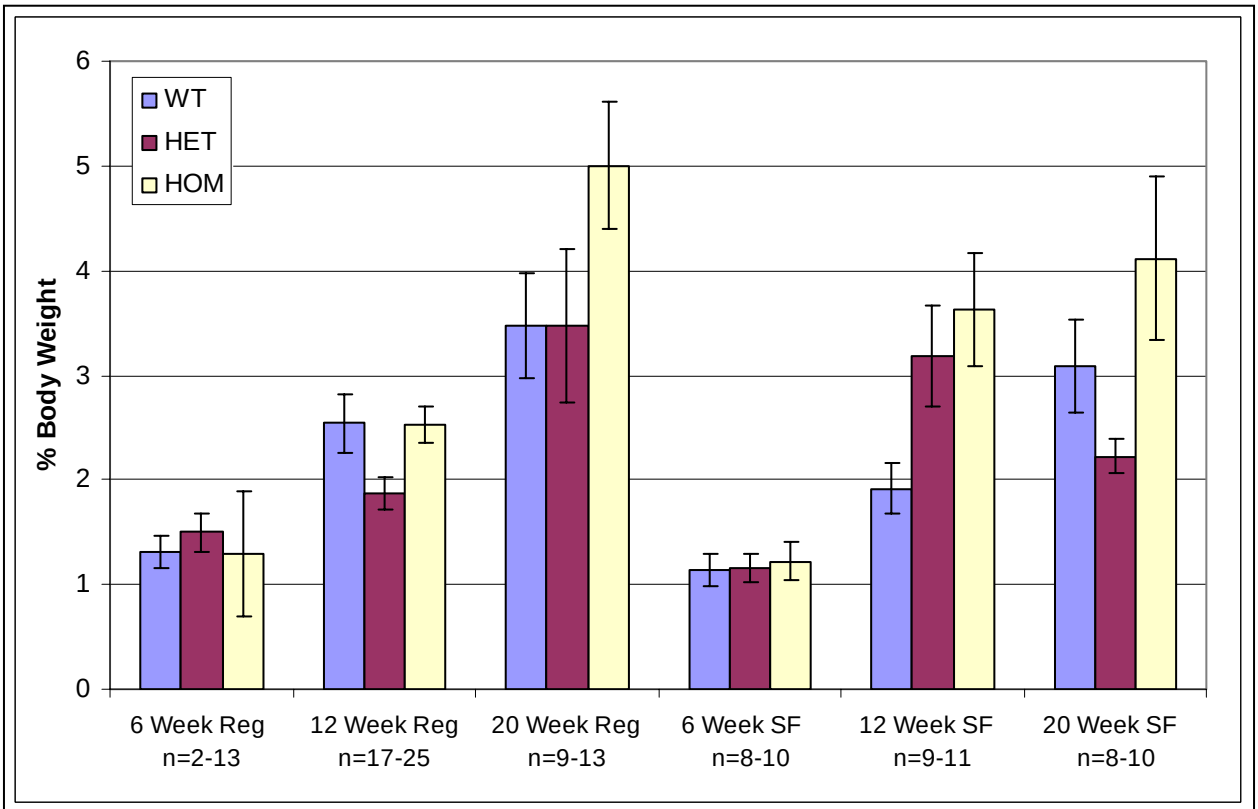

Figure 4. Female gonadel fat pad weights.
6-, 12-, and 20-week-old wild type (WT), heterozygous (HET), and homozygous (HOM) animals on a regular (Reg) and soy-free (SF) diet were analyzed.

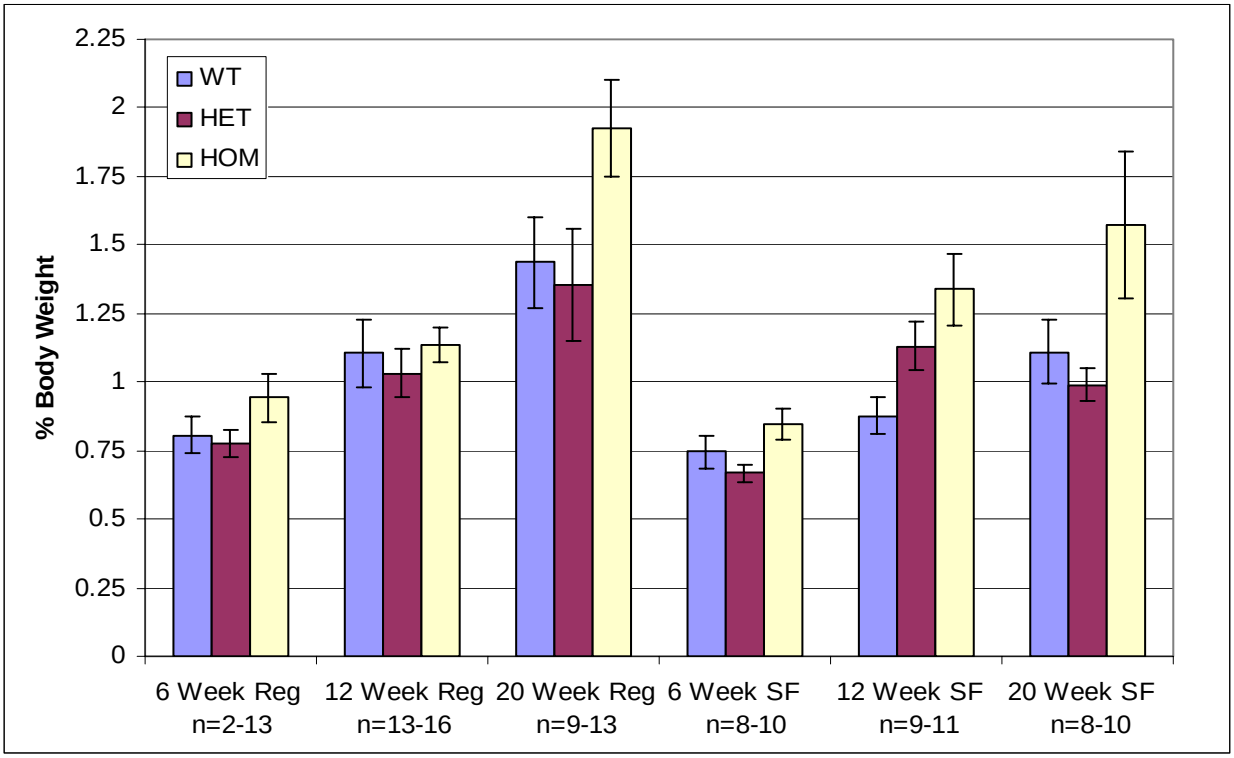

Figure 5. Female mammary fat pad weights.
6-, 12-, and 20-week-old wild type (WT), heterozygous (HET), and homozygous (HOM) animals on a regular (Reg) and soy-free (SF) diet were analyzed.

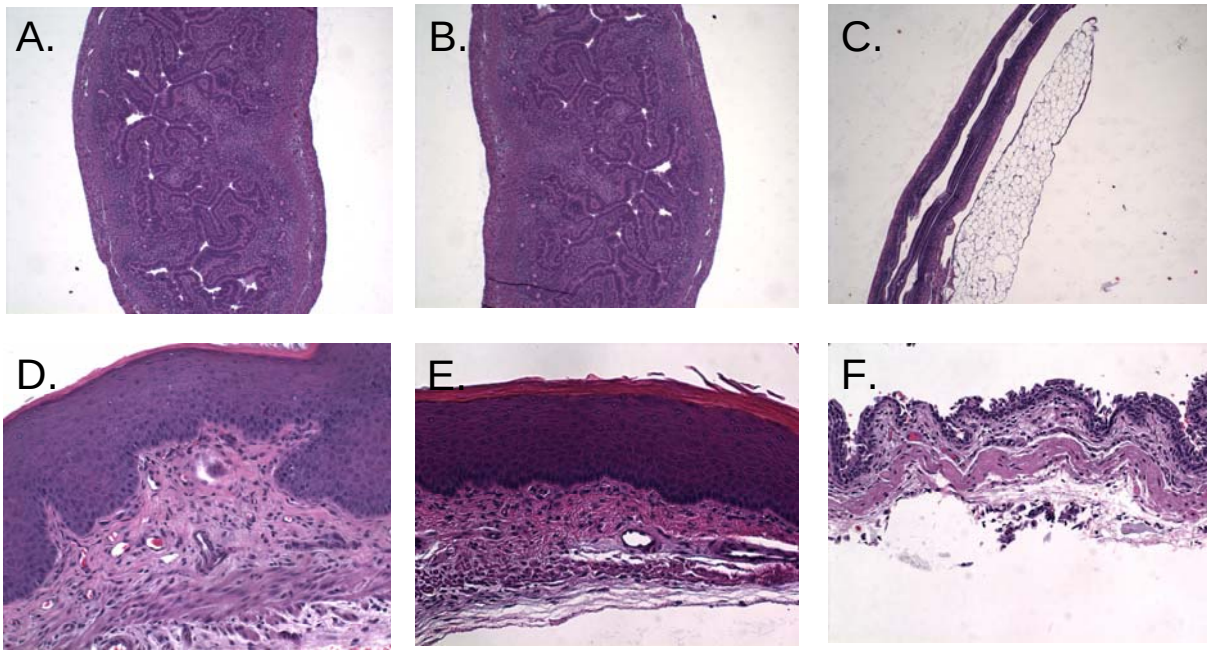

Figure 6. Uterine and vaginal histology of representative mice.
A-C: Uterine tissue hematoxylin and eosin (H&E) staining from 12-week-old wild type (A), heterozygous (B) and homozygous (C) mice (4x). Homozygous uterine tissues had immature and hypoplastic uterine tissue and a lack of estrogenization of the luminal and glandular epithelium. **D-F:** Vaginal tissue H&E staining from 12-week-old wild type (D), heterozygous (E) and homozygous (F) mice (20x). Homozygous vaginal tissues lacked the estrogen-induced stratification and cornification seen in the wild type and heterozygous tissues.

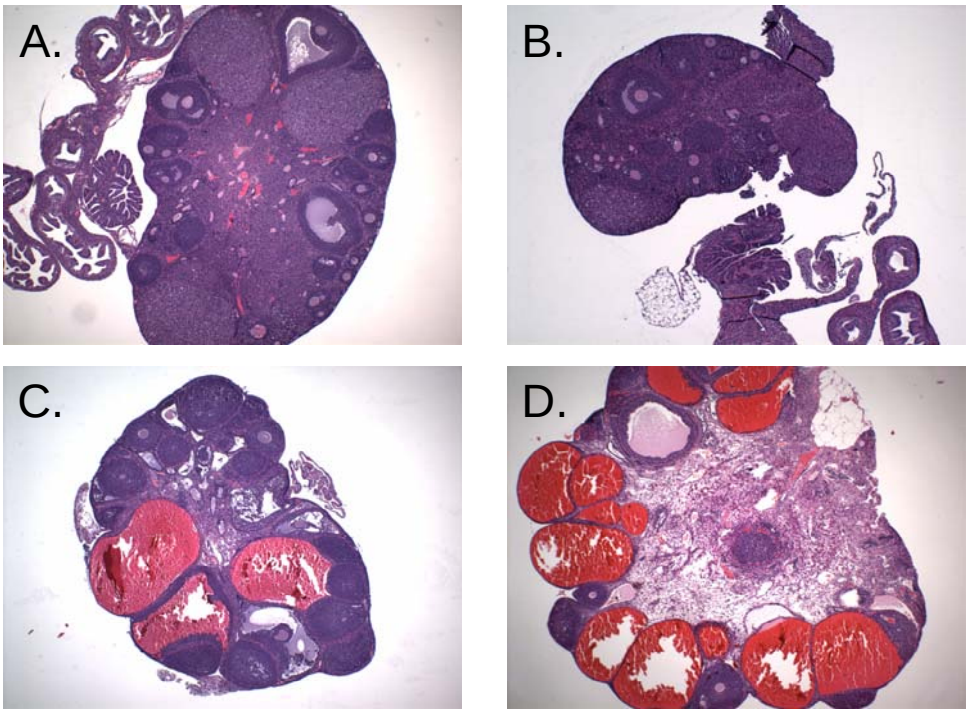

Figure 7. Ovarian histology of representative mice.
A-C: Ovarian tissue H&E staining from 12-week-old wild type (A), heterozygous (B) and homozygous (C) mice (4x). Homozygous ovaries had a hyperplasic stroma and no corpora lutea. In addition, some of the homozygous ovaries contained large, hemorrhagic, cystic follices. **D:** Ovarian tissue H&E staining from 20-week-old homozygous mice (4x). Severity of the homozygous phenotype increased with age.

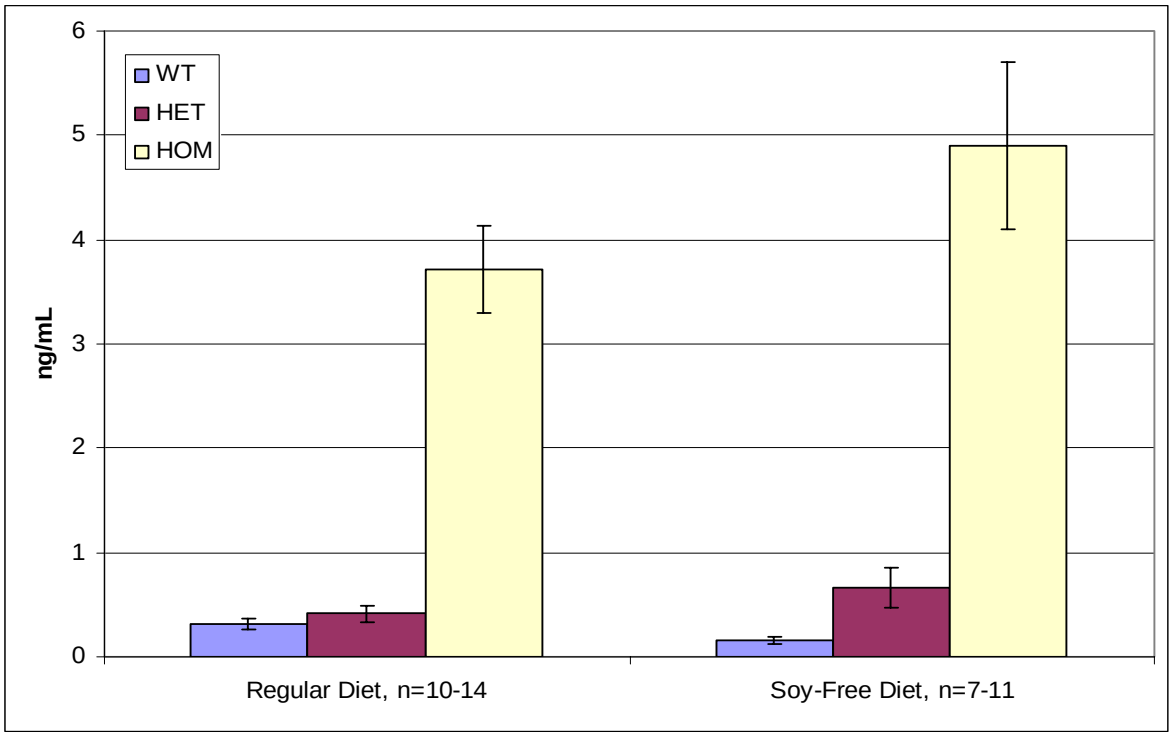

Figure 8. Female luteinizing hormone serum levels.
12-week-old wild type (WT), heterozygous (HET), and homozygous (HOM) animals on a regular and soy-free diet were analyzed.

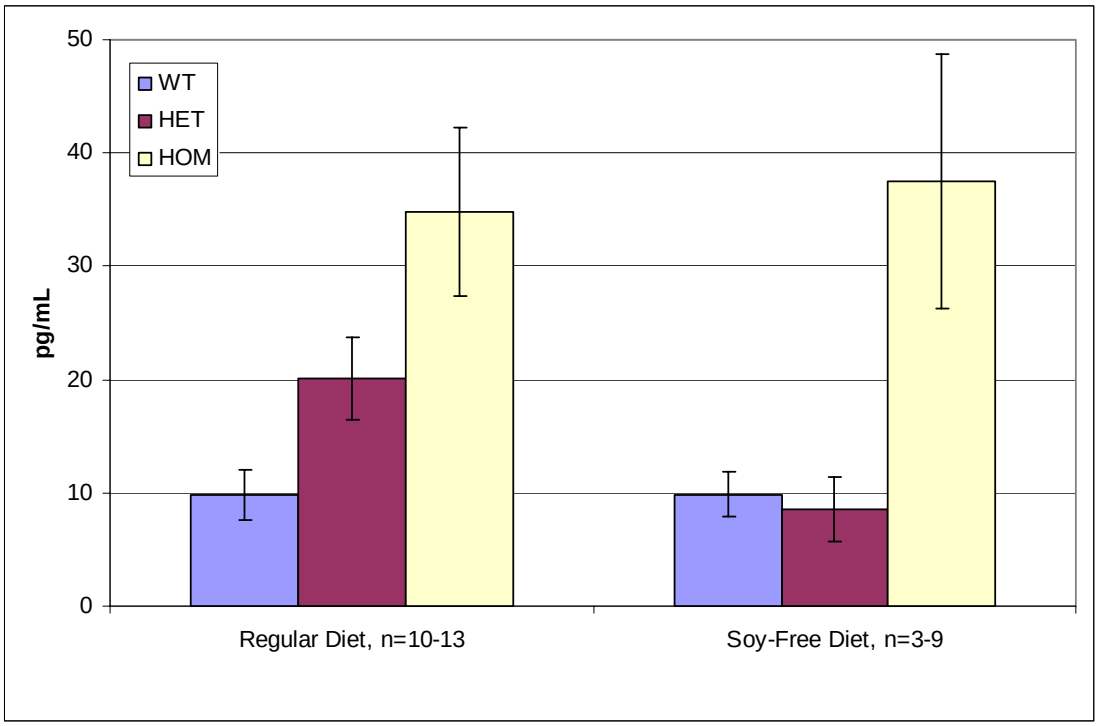

Figure 9. Female estradiol serum levels.
12-week-old wild type (WT), heterozygous (HET), and homozygous (HOM) animals on a regular and soy-free diet were analyzed.

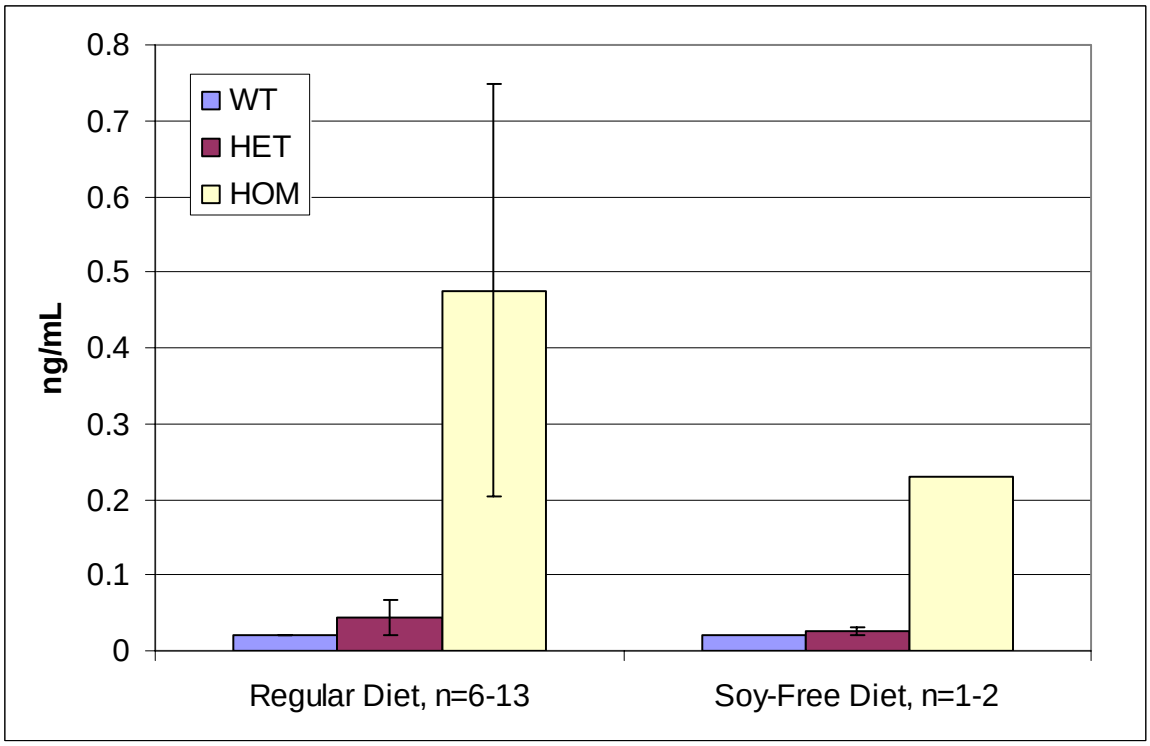

Figure 10. Female testosterone serum levels.
12-week-old wild type (WT), heterozygous (HET), and homozygous (HOM) animals on a regular and soy-free diet were analyzed.

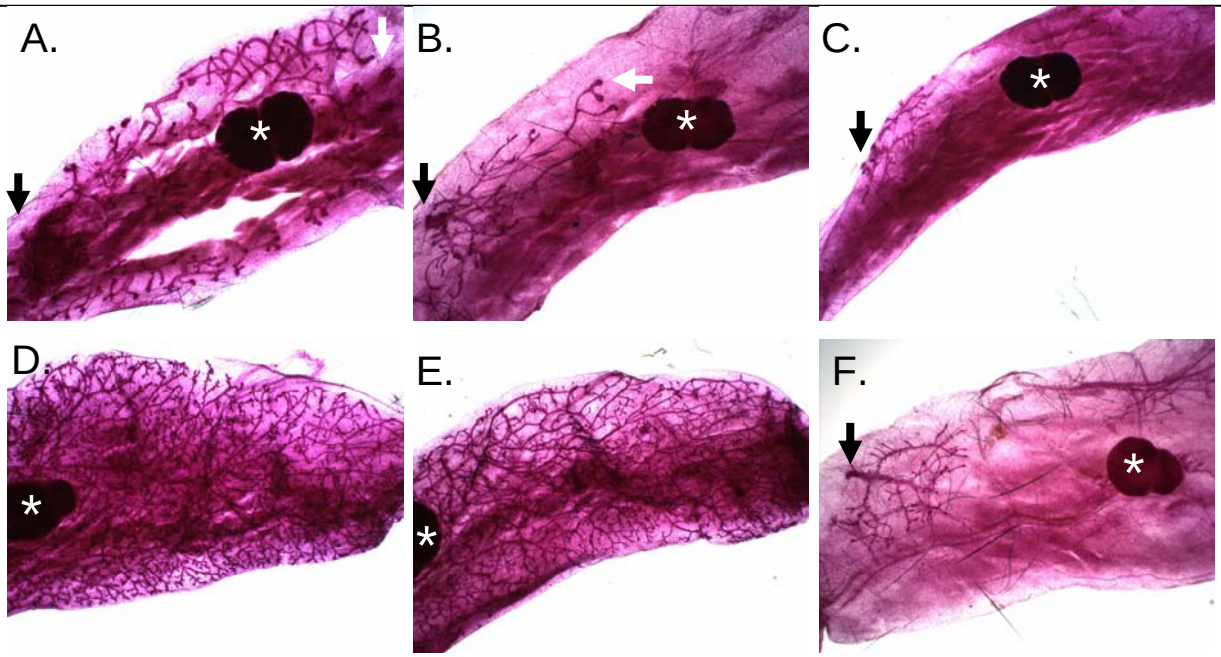

Figure 11. Mammary gland whole mounts of representative mice.
A-C: Mammary gland whole mounts from 6-week-old wild type (A), heterozygous (B), and homozygous (C) mice (1.25x). Wild type and heterozygous ductal trees extended to the lymph node (white asterisk) and had enlarged terminal end buds (white arrow), while homozygous mammary glands had a rudimentary epithelial ductal tree. Nipple location is indicated by the black arrow. **D-F:** Mammary gland whole mounts from 12-week-old wild type (D), heterozygous (E) and homozygous (F) mice (1.25x). Wild type and heterozygous ducts filled the entire mammary fat pad and had extensive branching and alveolar budding, while homozygous mammary glands did not develop beyond a rudimentary epithelial ductal tree.

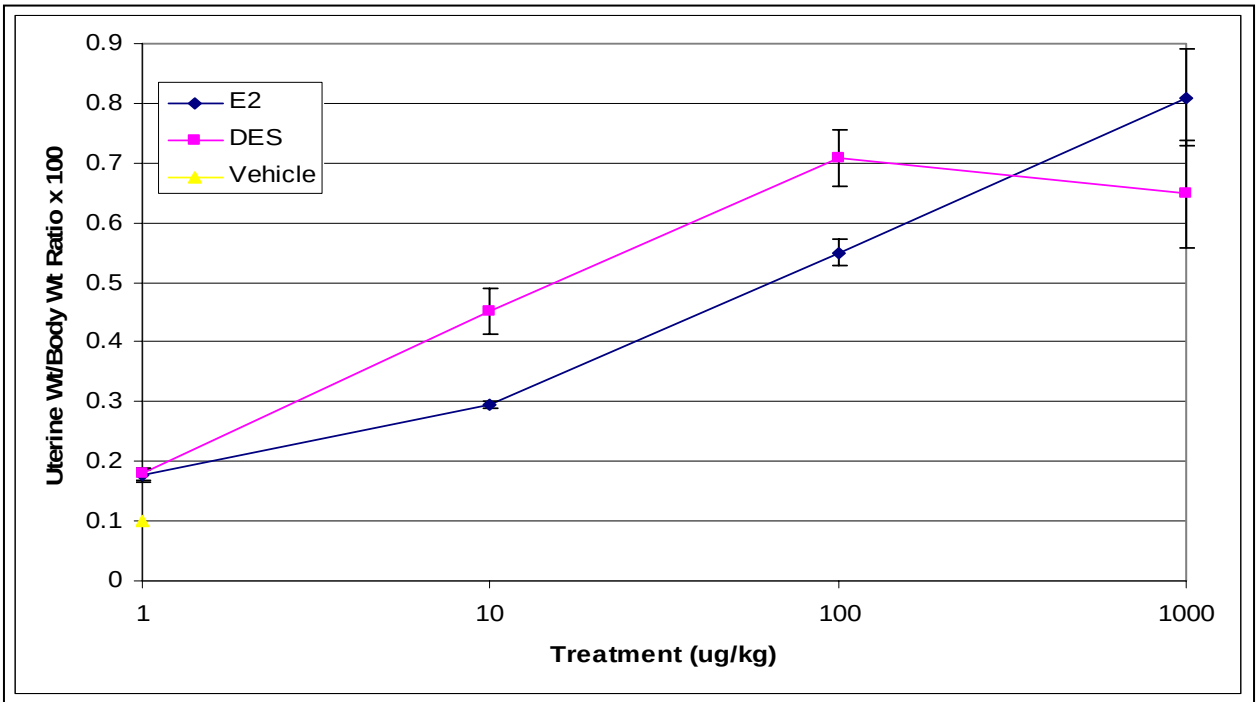

Figure 12. Uterotrophic assay results.
Wild type immature mice were subcutaneously injected with vehicle, estradiol (E2) or diethylstilbestrol (DES) at various doses for three consecutive days. Uterine wet weight was measured on the fourth day.

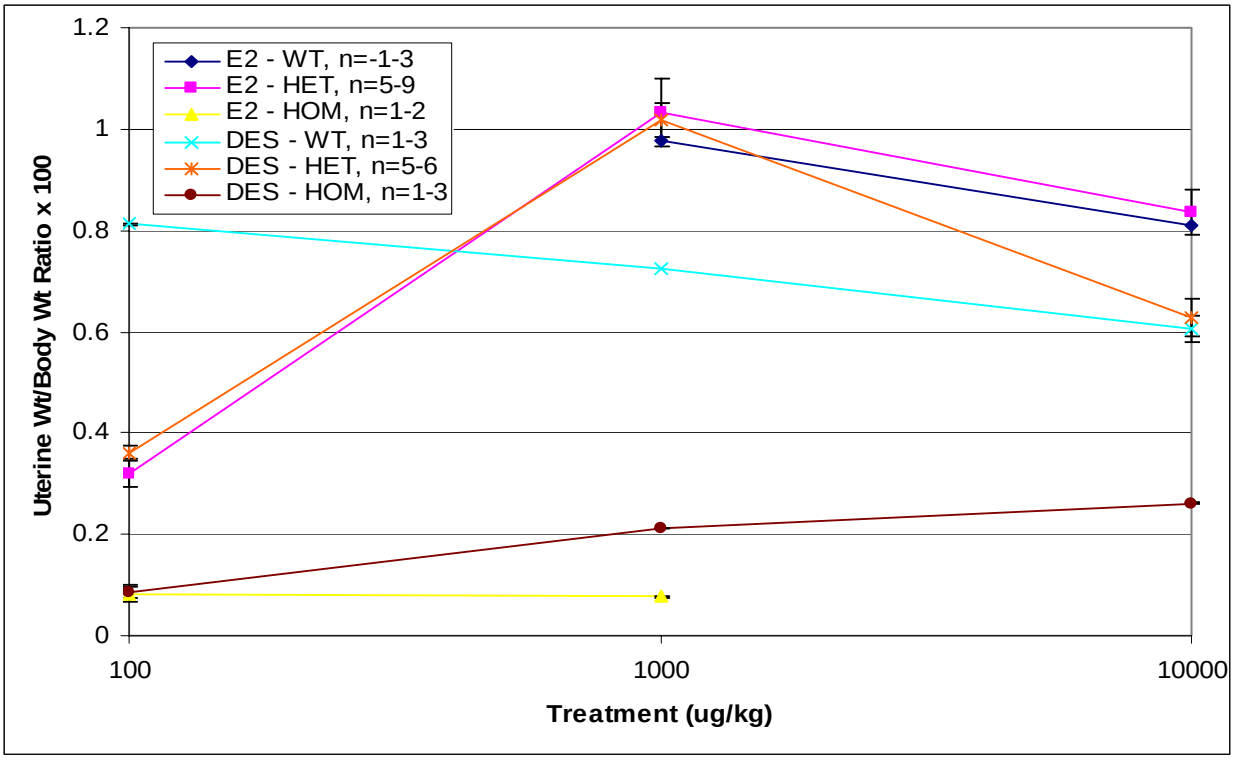

Figure 13. Uterotrophic assay results.
Wild type (WT), heterozygous (HET), and homozygous (HOM) immature mice were subcutaneously injected with estradiol (E2) or diethylstilbestrol (DES) at various doses for three consecutive days. Uterine wet weight was measured on the fourth day.